PHILIP MORRIS U. S. A.

PERSONAL AND CONFIDENTIAL

INTER-OFFICE CORRESPONDENCE

Richmond, Virginia

To: D. Leyden **Date:** January 31, 1992

From: R. Carchman, R. Kinser and R. McCuen

Subject: **1992 Operational Plans for the Tobacco Biochemistry Program**

The aim of the Program (as part of Strategic Goal #4 - Grow the Business Long Term) is to gather basic information relating to tobacco which will ultimately be applied to PM's new or existing products. This information will be forthcoming from the following areas using tobacco plants, cells and the cured leaf together with state-of-the-art technologies: precursor/product relationships; understanding the factors affecting the biological activity of cigarette smoke; and modifications to tobacco to affect its smoke composition and survivability during storage. These latter "focus areas" can be translated into the following mission statements:

for precursor/product - investigate how tobacco composition and cigarette construction affect smoke composition and quality;

for understanding the factors affecting the biological activity of cigarette smoke - develop an understanding of how cigarette construction, tobacco and smoke composition affect biological activity; and

for modifications to tobacco to affect its smoke composition and survivability during storage - modify tobacco to ascertain desirable agronomic and biochemical properties.

Richard Carchman (KSC)
Robert Kinser
Bob McCuen

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INVESTIGATE HOW TOBACCO COMPOSITION AND CIGARETTE CONSTRUCTION AFFECT SMOKE COMPOSITION AND QUALITY

Objective 1: To determine tobacco bound precursor/smoke product relationships for MS NNK by December 1992.

Introduction: While the amine precursor to MS NNN and NAT appears to be the appropriate tobacco minor alkaloid, the amine precursor of MS NNK has yet to be identified. Recently formulated hypotheses suggest the possibility of bound alkaloids or bound TSNA as NNK precursors.

Benefit: A cigarette with reduced levels of TSNA.

Strategy: Devise methods to reduce MS NNK formed from bound precursors during smoking.

Rationale: Previous work with water-washed filler and base web has shown that although preformed NNK levels are greatly reduced in these materials, the delivery of NNK into MS smoke is not significantly affected. It is therefore hypothesized that the MS NNK may arise from a bound form of NNK or a bound form of nicotine or other amines.

Status: At least three forms of bound nicotine have been identified, and a preliminary time course study of formation of bound nicotine was done in 1991. The rank order of NIC-Y in filler follows that of MS NNK, with burley being the highest, and oriental the lowest. Flash heating of ART burley filler previously depleted of soluble TSNA precursors resulted in release of additional NNK. In a separate experiment, mild base treatment of water washed DBC burley filler resulted in reduction of MS NNK delivery.

Tactics/Completion Dates/Responsible Individuals:

Determine whether there is a correlation between bound forms of nicotine and bound NNK during air-curing:

Using previously established procedures determine the levels of bound forms of nicotine (NIC-X, NIC-Y and NIC-Z) during air-curing of 1991 greenhouse and field grown burley 21 tobacco (2nd Q); S. Drew, P. Kurth.

Based on past studies of NNK stability in basic and acidic media, develop an extraction procedure to release and measure NNK from a bound form in burley tobacco (2nd Q); S. Drew, S. Haut, P. Kurth.

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Apply the extraction procedure to determine levels of bound forms of NNK during air-curing of 1991 greenhouse and field grown burley 21 tobacco (3rd Q); S. Drew, S. Haut.

Determine the levels of bound forms of nicotine (NIC-X, NIC-Y and NIC-Z) and NNK during air-curing of 1992 greenhouse and field grown burley 21 tobacco (4th Q); S. Drew, W. Hempfling.

Evaluate results from the air-curing study to determine the relationship between bound nicotine and bound NNK formation (4th Q); S. Drew, W. Hempfling.

Determine whether there is a correlation between bound forms of nicotine and the known rank order of MS NNK in fillers:

Complete analyses of bound nicotine (NIC-Y and NIC-Z) in water-washed burley, bright and oriental fillers (2nd Q); S. Hassam, P. Kurth, R. Kaiser.

Measure levels of bound nicotine (NIC-X, NIC-Y and NIC-Z) in Japanese fillers (3rd Q); R. Forte, P. Kurth.

Isolate a bound form of nicotine and determine its structure:

Develop isolation methods for a bound form of nicotine (e.g. NIC-X) *via* chromatography of burley extracts (3rd Q); R. Izac, W. Hempfling.

Develop a means of isolation of a bound form of nicotine by acid digestion of marc from water washed burley. (3rd Q); R. Izac, W. Hempfling, P. Kurth.

Determine the chirality of nicotine in its bound form and whether there is any relationship to the chirality of nicotine in smoke:

Determine the chirality of nicotine liberated by alkaline digestion of burley filler (4th Q); R. Izac, P. Kurth.

Synthesis of bound nicotine based on the hypothesis that nicotine is chemically bound to lignin:

Develop a non-enzymatic means of synthesizing a bound form of nicotine from nicotine and known phenolics (3rd Q); R. Izac.

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Investigate enzyme-catalyzed synthesis of a bound form of nicotine from nicotine and phenylpropanoids (3rd Q); S. Hassam, W. Hempfling.

Study the release of NNK from bound NNK sources using chemical and flash heating methods; determine if there is a correlation between bound nicotine and bound NNK in the same sources:

Develop a pyrolytic method for liberation of NNK from water-extracted burley fillers (2nd Q.); W.R. Morgan, R. Kaiser, P. Kurth.

Apply the flash heating method to determine release of NNK from water-extracted bright fillers (2nd Q); W.R. Morgan, R. Kaiser.

Develop a method for flash heating MS TPM to determine if TSNA precursors are present in TPM (4th Q); W.R. Morgan, R. Kaiser.

Repeat a 1991 study to determine alkali- and acid-releasable nicotine from water-extracted ART burley filler (3rd Q); R. Hellams, P. Kurth.

Develop a means to enrich water-extracted ART burley filler in bound nicotine and NNK by using N-methylmorpholine-N-oxide (4th Q); R. Hellams, W. R. Morgan.

Apply flash heating method to determine release of NNK from ART Bu filler from the above studies (when available); W.R. Morgan.

Investigate the oxidation and nitrosation of synthetically bound nicotine, followed by release of NNK via pyrolysis or alkaline digestion (4th Q); W.R. Morgan, R. Izac.

Resource Allocations:

Scientist - 1.4 Man Year
Research Scientist - 2.9 Man Year
Applied Research Director Staff (Assoc. Prin. Scientist) - 0.1 Man Year
Technician III - 1.2 Man Year
Greenhouse Personnel - 0.1 Man Year
Piedmont South Experimental Station, Blackstone, Va. - <0.1 Man Year (supply 6 field-grown Bu 21 plants)
ARD - <0.1 Man Year

Objective 2: To design a model cigarette with reduced MS TSNA delivery by the end of 1993.

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Introduction: In past research, some MS TSNA precursors have been identified, and methods have been investigated to remove preformed TSNA and/or precursors from filler. These methods have been successful in reducing MS NNN and NAT, but not NNK. Accomplishment of Objective 1 will provide further information regarding MS NNK precursors, particularly bound precursors. The focus of Objective 2 is to investigate reduction of MS TSNA delivery by physical removal of TSNA from filler, by varying cigarette construction parameters and by using naturally occurring low TSNA tobaccos.

Benefit: A cigarette with reduced levels of TSNA.

Strategy 1: Reduce MS TSNA by modification of cigarette construction parameters.

Rationale: Past studies have been varied, and lack a systematic approach to study the interactive effects of construction parameters on MS TSNA delivery.

Status: A proposal was developed to use response-surface methodologies to evaluate, in a systematic manner, construction parameters and TSNA delivery. Cigarettes for study have been designed and are being manufactured by Semi-works personnel.

Tactics/Completion Dates/Responsible Individuals:

Systematic response-surface study of cigarette construction parameters:

Fabrication by Semi-works personnel of 30 types of model cigarettes with different construction parameters (blend components, paper porosity and paper additives, ventilation and filter efficiency) (1st Q); R. Hellams.

Determine MS TSNA/dry TPM and obtain CTSD data for the above cigarettes (2nd Q); R. Hellams, R. Kaiser, P. Kurth.

Determine MS TSNA/dry TPM and obtain CTSD data for the above cigarettes with plugs pulled/holes taped (2nd Q); R. Hellams, R. Kaiser, P. Kurth.

Evaluate data for use as a predictor for MS TSNA delivery of a given cigarette (2nd Q); R. Hellams.

Strategy 2: Reduce MS TSNA by selective physical removal of TSNA and TSNA precursors.

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Rationale: Extraction of fillers with water or ethanol or by SCFE has been shown to reduce preformed TSNA and thereby reduce delivery of MS TSNA. However, while the delivery of MS NNN and NAT is greatly reduced, the delivery of MS NNK is unchanged. Exogenous nitrates and nitrites have been shown to increase TSNA delivery.

Status: A feasibility study by ARD using supercritical CO_2 modified with triethylamine showed that nornicotine is removed from burley filler. An SCFE instrument has been acquired. Electrodialysis has been shown to remove nitrates from tobacco solubles.

Tactics/Completion Dates/Responsible Individuals:

Physical removal of TSNA and precursors from filler:

Optimize a method using supercritical CO_2 to remove TSNA and secondary amine alkaloids (2nd Q); S. Haut, R. Forte, R. Kaiser, P. Kurth.

Develop a method using supercritical CO_2 modified with a polar additive to remove TSNA and secondary amine alkaloids (4th Q); S. Haut, R. Forte.

Reformulate the reduced TSNA water-extracted filler to have desirable subjective and burn properties (4th Q); S. Haut, R. Kaiser, P. Kurth.

Determine the effect of removing nitrate from Bu S1 fraction of Bu CEL via electrodialysis on MS TSNA (3rd Q); S. Drew, S. Haut, P. Kurth.

Strategy 3: Reduce MS TSNA by use of tobaccos naturally low in preformed TSNA, minor alkaloids and/or nitrosating agents.

Rationale: Fillers from which alkaloids and preformed TSNA have been removed show a reduced delivery of MS TSNA. Exogenous nitrates and nitrites have been shown to lead to increased delivery of MS TSNA.

Status: TSNA analysis of different burley grades is in progress.

Tactics/Completion Dates/Responsible Individuals:

Investigate the possibility of utilizing tobaccos which are naturally low in production of MS TSNA:

Complete preformed and MS TSNA analysis of burley grades from 1988, 1989, 1990 crop years and determine whether there are individual grades that make significant contributions to MS TSNA of the blend (1st Q); S. Haut.

Strategy 4: Reduce MS TSNA by use of tobacco genotypes, agronomic conditions and curing regimens that would result in tobacco plants naturally low in preformed TSNA, minor alkaloids and/or nitrosating agents.

Rationale: Oriental, Japanese and some South American tobaccos are naturally lower in TSNA than American grown burley or bright tobacco. However the growing and curing conditions for these tobaccos are not always known precisely. Past studies have shown that the chemical composition (including TSNA levels) of bright, burley and oriental tobaccos can be affected by agronomic conditions and curing regimens. Climatic factors such as rainfall, soil type and diurnal light levels may also affect TSNA and/or precursor levels in the tobacco plant. Past studies lack a systematic approach to study soil and climatic factors that affect TSNA levels. Past studies have shown that fillers from which alkaloids and preformed TSNA have been removed show a reduced delivery of MS TSNA. Exogenous nitrates and nitrites have been shown to lead to increased delivery of MS TSNA.

Status: Past studies have shown that Oriental tobaccos grown in Turkey, Greece and Asia differ in TSNA levels. Flue-cured and burley tobaccos grown in Japan have lower levels of preformed and MS TSNA than comparable U.S. grown tobaccos, even though alkaloid and nitrate levels are comparable. Field studies have been done to measure component changes, including TSNA.

Tactics/Completion Dates/Responsible Individuals:

Investigate the effect of light, soil and other agronomic conditions on TSNA and precursor levels in tobacco and MS smoke:

Initiate a greenhouse or growth chamber study to determine the effect of light, fertilization, water and soil on TSNA and precursors in burley tobacco (Bu21) (2nd Q); S. Haut, R. Kaiser, greenhouse personnel.

Harvest, cure and analyze leaves from the above study (4th Q); S. Haut, R. Kaiser, P. Kurth.

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Resource Allocations:

Scientist - 0.6 Man Year
Research Scientist - 2.1 Man year
Applied Research Director Staff (Assoc. Prin. Sci.) - 0.1 Man Year
Technician III - 0.8 Man Year
Greenhouse Personnel - <0.2 Man Year
Semi-Works (30 different codes of cigarettes; 4,000 cigts. per code; total = 120,000 cigts.)
CTSD - (30 different codes of cigts.)
ARD - 0.1 Man Year
Statistician - <0.1 Man Year

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DEVELOP AN UNDERSTANDING OF HOW CIGARETTE CONSTRUCTION, TOBACCO AND SMOKE COMPOSITION AFFECT BIOLOGICAL ACTIVITY

Objective 1: To quantify the effect of cigarette construction parameters and specific additives on the biological activity of CSC by the end of 1992.

Introduction: Various consumer-health groups have attempted to link cigarette smoke with a variety of human disorders. The scientific community has issued several documents relating to smoking and health; e.g., the Banbury Report #3 - A Safe Cigarette?, various U.S. Surgeon General's Reports, etc. As an industry, we must be in a position to address these and future allegations pertaining to our product.

Benefit: Increased consumer acceptability of the product.

Strategy 1: Determine the interactive effects of modifications to conventional cigarette construction on the S/M activity of CSC.

Rationale: A systematic study of selected construction parameters will show which ones are related to decreasing bioactivity.

Status: Previous non-systematic studies have suggested that CSC from a low tar reference cigarette was more active than CSC from the Kentucky Reference cigarette. But the low tar reference cigarette had a high efficiency cellulose acetate filter, high porosity paper and a 47% filter dilution. Other studies suggested that filter dilution was a critical factor in determining bioactivity. Meetings have been held with the appropriate groups to determine what cigarette models to make and how to make them. Thirty models are being prepared in the Semi-works.

Tactics/Completion Dates/Responsible Individuals:

In collaboration with INBIFO personnel, determine the interactive effects of blend components, paper porosity and paper additives, ventilation and filter efficiency on the S/M activity of 30 model CSCs (2nd Q); R. Hellams; F. Tewes.

Identify further cigarette construction parameters (e.g., different filters and various cigarette circumferences) for part 2 of the above study (3rd Q); R. Hellams.

Investigate peak coal temperature of a cigarette *versus* S/M activity of the CSC (3rd Q); R. Hellams; F. Tewes.

Resource Allocations:

Research Scientist - 0.1 Man Year
INBIFO personnel - 0.15 Man Year

Objective 2: To monitor the external scientific literature and attend relevant meetings for new developments relating to biological activity; propose new studies for PM USA R&D and INBIFO personnel when appropriate information exists (Ongoing).

Rationale: Scientific studies in a variety of areas will be useful in understanding the biological activity of smoke.

Status: This is an ongoing effort with PM USA R&D personnel and those individuals at INBIFO.

Tactics/Completion Dates/Responsible Individuals:
Use Dialog™ on a biweekly basis to search the relevant external scientific literature for articles of interest; circulate search results to appropriate personnel (Ongoing); C. Gregory, R. Pages, R. Carchman, G. Patskan.

Resource Allocations:

Research Scientist - 0.3 Man Year
Senior Scientist - 0.1 Man Year
INBIFO personnel - 0.25 Man Year
Technical Information Section personnel - 0.25 Man Year

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MODIFY TOBACCO TO AFFECT ITS SMOKE COMPOSITION AND SURVIVABILITY DURING STORAGE - MODIFY TOBACCO TO ASCERTAIN DESIRABLE AGRONOMIC AND BIOCHEMICAL PROPERTIES

Objective 1: Modify tobacco plants so that they produce reduced levels of alkaloids as compared to the cultivars currently in use by December, 1995 (PMT-based modification, December, 1993 and an MPO-based modification, December, 1995).

Introduction: Nicotine and other minor alkaloids impact the quality of smoke in cigarettes. By lowering the level of these alkaloids in tobacco, the quality of smoke may be improved. This program was initiated as a proof-of-concept study. Its success will lay the foundation of biotechnology at R&D.

Benefit: This product will bring key technology to the PM Research Center. Such technology will be applicable to the solution of many problems and generation of new products. Successful achievement of this objective will result in a low alkaloid cigarette.

Strategy 1: Influence tobacco biochemistry by expressing the antisense of the cDNA sequences that are overly expressed in tobacco root to reduce the level of alkaloids in tobacco plants.

Rationale: Alkaloids are synthesized in the tobacco root and transported to the leaf. Enzymes involved in alkaloid biosynthesis are coded for by genes that are expressed in the tobacco roots. The genes that are overly expressed in tobacco root will include genes involved in alkaloid metabolism. Such genes, depending upon the level of expression, may be isolated by differential hybridization. The DNA sequences of these tobacco root specific genes can be expressed in tobacco plant in antisense orientation to counteract the expression of the resident genes. Some of these genes when expressed in antisense orientation may decrease the level of alkaloid biosynthesis by decreasing the level of the enzymes involved in alkaloid biosynthesis.

Status: Ten clones have been isolated which contain DNA from genes that are overly expressed in tobacco root. A vector that can be used to express genes in tobacco has also been constructed. This vector has been used to clone the DNA from the ten genes in the antisense orientation. Some of these antisense constructs have been inserted into tobacco tissue which has been regenerated into transgenic plantlets. Some of these transgenic plantlets have been transferred to the greenhouse. Several additional plantlets are at various phases in the same process.

Tactics/Completion Dates/Responsible Individuals:

The transgenic plants will be examined for their individual alkaloid production profile (On-going); R. Izac.

Additional transgenic plants will be created as antisense constructs of interest become available (On-going); M. Shulleeta, B. Vaughan.

Strategy 2: Express the antisense of the DNA sequences for PMT in tobacco plants.

Rationale: Expression of the DNA sequences for PMT (putrescine N-methyltransferase) in the antisense orientation in tobacco plants will compete with the expression of the resident homologous gene and thereby decrease the level of PMT in the tobacco root. Diminished level of enzyme should result in decreased alkaloids in the tobacco plant.

Status: Protein preparations with significant enrichment of PMT activity have been generated. A band on SDS-PAGE has been tentatively identified as PMT. Amino acid sequence for two regions of PMT has been generated. Degenerate oligonucleotide primers based on the amino acid sequence have been synthesized.

Tactics/Completion Dates/Responsible Individuals:

Prepare samples and obtain additional amino acid sequence for PMT protein with an outside contractor (2nd Q); H. Nakatani.

Use the amino acid sequence information to synthesize additional oligonucleotide primers for use in PCR (2nd Q); S. Wahab, D. Turner, V. Malik.

Use PCR to isolate desired sequences from the tobacco genomic DNA and tobacco root mRNA (3rd Q); V. Malik, S. Wahab, D. Turner, T. Michalik.

Develop procedures to screen polyclonal antibodies that have been produced against the synthetic peptide (2nd Q); V. Malik, B. Davies.

Use antibodies to purify PMT (3rd Q); H. Nakatani, M. Krauss, B. Davies.

Obtain amino acid sequence of the PMT purified by use of an antibody with an outside contractor (3rd Q); H. Nakatani.

Obtain the base sequence of cDNA fragments obtained by PCR (4th Q); T. Michalik, S. Wahab, V. Malik.

Conduct PMT assays for screening of transgenic plants, as required, including harvesting and processing (4th Q); J. Lyle, S. Davies, B. Vaughan.

Strategy 3: Reduce the level of nicotine in the tobacco plant by affecting the enzyme pathway through manipulation of MPO.

Rationale: PMT and MPO (N-methylputrescine oxidase) are two enzymes involved in the biosynthesis of nicotine (pyrrolidine ring). The final ring structure is formed non-enzymatically. To lower the nicotine level completely may require regulation at both enzyme sites in this pathway.

Status: Appropriate substrates are available to initiate this study. For completion, further synthesis of substrates are required (non-radiolabeled and radiolabeled).

Tactics/Completion Dates/Responsible Individuals:

Examine and refine MPO enzyme assay (radiochemical) method/examine non-radioactive protocol. Obtain operational assay protocol (1st Q); S. Davies, H. Nakatani.

Modify assay protocol, optimize, alter and obtain SOP (4th Q); S. Davies, T. Yu, H. Nakatani.

Obtain partially purified MPO preparations from tobacco root materials grown in hydroponic cultures (process through ammonium sulfate fractionation, 40-65% saturation) (continuing); J. Lyle, S. Davies, B. Vaughan, greenhouse personnel.

Examine partially purified MPO preparations (ammonium sulfate stage) for characterization studies to assist in purification efforts, e.g., pH optimum, pI, etc.; continue characterization during purification (3rd Q); S. Davies, T. Yu.

Purification of MPO

Apply classical column chromatography to ammonium sulfate fractionated material: phenyl-Sepharose, anion exchange for use in purification of MPO (3rd Q); J. Lyle, S. Davies, T. Yu.

Examine metal chelate chromatography for possible use in purification of MPO (4th Q); M. Penn, H. Nakatani.

HPLC (hydrophobic interaction, ion exchange) (4th Q); H. Nakatani, T. Yu.

2-D examination (initiate 3rd Q and continuing); M. Krauss.

Examine enzyme stability/activity staining methodologies/spectroscopy (4th Q); T. Yu, H. Nakatani, M. Penn, S. Davies.

Design effectiveness of chromatographic steps for inclusion in series and bulk process (4th Q); H. Nakatani, T. Yu, S. Davies, J. Lyle, M. Penn.

Acquire Waters 650 System in-house to assist in preparative purification methods. Develop/use in more efficient purification of MPO through fast flow system (continuing); J. Lyle, H. Nakatani.

Evaluate HPCE technologies and other technologies for purification of MPO. Acquire HPCE system in-house to assist in protein purification (obtain 4th Q); H. Nakatani.

Strategy 5: Reduce the level of nicotine by affecting the biosynthetic pathway at nicotine synthase.

Rationale: Nicotine is formed by conjugation of the pyrrolidine ring (formed through MPO and PMT and putrescine) and nicotinic acid via an enzyme, nicotine synthase. With MPO, PMT and nicotine synthase, the *complete* biosynthetic pathway for nicotine formation can be manipulated.

Status: This enzyme is assumed to exist from literature examination. (Friesen, J. B.; Leete, E. Nicotine synthase - an enzyme from *Nicotiana* species which catalyzes the formation of (S)-nicotine from nicotinic acid and 1-methyl-Δ'-pyrrolinium chloride. Tetrahedron Letters. 31: 6295-6298). No work in-house has been conducted directly with this enzyme.

Tactics/Completion Dates/Responsible Individuals:

Examine and develop an enzyme assay protocol including obtaining necessary substrates (Initiate 1992, Complete 1993); M. Steele, W. Hempfling.

Verify presence of nicotine synthase in root extracts upon receipt of synthesized substrates (initiate, 2nd Q, 1992; complete 1993); M. Steele, W. Hempfling, J. Lyle.

Optimize enzyme assay conditions with respect to pH, temperature, concentration of substrates, potential activators and inhibitors (Initiate 1992, Complete 1993); M. Steele.

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Decision point on further research based upon the above (complete 1992); W. Hempfling, M. Steele.

Resource Allocations:

Senior Scientist - 1.0 Man Year
Associate Senior Scientist - 1.0 Man Year
Research Scientist - 1.85 Man Year
Scientist - 1.25 Man Year
Associate Scientist B. - 4.25 Man Year
Technician IV - 1.0 Man Year
Technician III - 1.0 Man Year
Applied Research Directorate Staff (Assoc. Prin. Sci.) - 0.25 Man Year
Greenhouse personnel - 0.75 Man Year
Medical College of Wisconsin

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/mps

cc: D. Ayers
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C. Ellis
S. Hassam
V. Malik
H. Nakatani
G. Nixon
G. Patskan

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